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Bace1 and Neuregulin-1 (Nrg1) cooperate to control formation and maintenance of muscle spindles

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: David del Alamo

1st Editorial Decision 22 February 2013

Thank you for the submission of your manuscript to The EMBO Journal. It has been sent to three referees, and we have so far received reports from two of them, which I copy below. As both referees are convinced about the interest and quality of your study, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this is a preliminary decision made in the interest of time, and I will forward you the third report, probably including further requests, as soon as I receive it.

Without going into details that you will find below, both referees are very positive and ask mainly for minor text and technical clarifications. Please be aware that it is 'The EMBO Journal' policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will essentially depend on the completeness of your responses included in the next version of the manuscript

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process iniciative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact me as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let me know in advance and we may be able to grant an extension.

Do not hesitate to contact me by e-mail or on the phone in case you have any questions or need further input.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

Here, Cheret et al. report that BACE1 processing of IgNrg1β1 is required for formation, maturation, and maintenance of muscle spindles. The authors perform a series of experiments with BACE1-/- mice, BACE1 inhibitor treatment of wild-type mice, and conditional Nrg1 mice that are analyzed by gait analysis, biochemistry, and histology. The authors conclude that Bace1 plays a role in coordinated movement through regulating muscle spindle physiology and that an unwanted side effect of Bace1 inhibition for AD might be impaired coordination.

This is an interesting report with significant implications for both the AD and proprioception fields.

Comments:

1) In Figs. 1C-E and 5C-E, hindlimbs appear to be more severely affected than forelimbs in the various BACE1 and Nrg1 null mice. This might result from hypomyelination rather than spindle dysfunction, at least in the BACE1 knockout. Because hindlimb myelinated motor axons are longer than those of forelimbs, hindlimb motor axons would be expected to be more affected by hypomyelination than forelimbs. Although the authors measured g-ratios of sciatic nerve axons, representative images should also be inclided. In addition, the authors need to proved evidence that the gait disturbances are directly related to spindle dysfunction rather than hypomyelination in the mutants, particularly in the BACE1 knockout where hypomyelination is well established.

2) Include age of adult mice and dose treated with BACE1 inhibitor in the text and the legend of Fig. 1I-K.

3) Fig 3F-H: black bars are control mice but the key in G indicates white bars are controls.

4) For the conditional Nrg1 knockout coTxNrg1 (Fig 4.), evidence of lack of Nrg1 expression in muscle spindles is not shown but should be included.

5) Fig. 4D: How do the authors explain that the % of 1500-2000um spindles is significantly higher in coTxNrg1 mice? The % of spindles >2000um is clearly decreased, indicating spindle degeneration, but the increase in 1500-2000um spindles is confusing. It would be less confusing if the authors plot total number of spindles in each size range in addition to %.

6) The cre transgenic mice used in the study are not well described. Are the Deletercre animals mentioned in the materials and methods the same as the Wnt1cre mice mentioned in the results? When is the cre transgene expressed in the Wnt1cre mice? What promoter used and the tissue expression profile for the Cre-ERTM mice?

7) Are the phenotypes the same for co-IgNrg1 and coTxNrg1 mice? A description of where the loxP sites are located in Nrg1 gene for the coTxNrg1 mice is lacking. It is assumed that coTxNrg1 is a total null of all Nrg1 isoforms, but this is not explicit. This is an important point, because there may be important differences between complete Nrg1 and IgNrg1-only null mice.

8) Abstratct: "Our results should allow to monitor the effects of Bace1 inhibition in vivo." It is unclear exactly what the authors mean by this.

Referee #3

This manuscript showed that Bace1 processed IgNrg1b1 in in vitro proteolysis assay and suggests that Bace1 dependent processing of IgNrg1b1 is necessary for formation and maturation of muscle spindles in mice. This work, using a variety of molecular and mouse genetic approaches, provides strong evidence for their claims. While no major concerns exist, several minor points need to be addressed.
1. The authors stated that Bace1-dependent proteolysis is rate-limiting for spindle-inducing activity of IgNrg1. In experiments that showed increase of 50% of muscle spindles in IgNrg1b1ov transgenic mice would suggest that Bace1 is not rate-limiting. This point should be made clearer.
2. The authors showed that ubiquitous deletion of Nrg1 in coTxNrg1 mice exhibit in addition to less number of spindles, outer capsule degeneration, a phenotype that is not observed in Bace1-/- mice, suggesting that Bace1 and Nrg1 act independently in controlling muscle spindle physiology. Does coTxNrg1 mice showed that same coordination defects as observed for Bace1-/- mice? Could Bace1 and Nrg1 signaling pathway be acting in separate pathways?

Additional Correspondence 26 February 2013

Thank you again for the submission of your research manuscript to The EMBO Journal. As I mentioned in my previous letter, your manuscript was sent to three referees and we have just received the third report, which I copy below.

As you will see, referee #2 is slightly more negative than referees #1 and #3, but still considers your work interesting and recommends its publication in The EMBO Journal once some concerns and suggestions are addressed as explicitly explained in his/her report.

Do not hesitate to contact me by e-mail or on the phone in case you have any questions or need further input.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORT

Referee #2

It was previously shown that Nrg1 is required in the early stages of muscle spindle development (Hippenmeyer et al., 2002). Between E14.5 and E18.5 proprioceptive afferents (Parvalbumin+ fibers) are required to induce the expression of early markers of muscle spindle differentiation. In Isl1Cre/Nrg1flox/- mice myofibers fail to express these markers. It was further shown that Ig-Nrg1 isoforms are preferentially expressed by proprioceptive sensory neurons and are sufficient to induce muscle spindle differentiation in vivo, whereas CRD-Nrg1 isoforms not required for muscle spindle induction.

The present manuscript by Cheret and coworkers confirms and extends these observations using a novel conditional Nrg1 mouse line in which the Ig-like domain of Nrg1 was targeted by Cre/loxP recombination. In addition, the present work reports on a new role of Bace1 in muscle spindle formation during development and In their maintenance in the adult. A tamoxifen-inducible Nrg1 KO was used to demonstrate a role for Nrg1 in maintenance of muscle spindles. Ablation of Bace1 and IgNrg1 produced similar gait abnormalities. In vivo overexpression of IgNrg1 in axons increased muscle spindle numbers, a defect which was rescued in the absence of Bace1, consistent with a model that Bace1-dependent cleavage of Nrg1 controls muscle spindle formation.

Overall, the technical quality of the data is excellent; the conceptual advance over previous work is decent, but not exceptional; the mechanistic evidence for Bace1- dependent cleavage of Nrg1 controling muscle spindle formation is modest.

Major points:
1. Bace1 has many substrates in neurons. A recent study reported 34 substrates in primary

embryonic neurons (Kuhn et al., 2012) and Nrg1 was not even included suggesting that this list is not complete. Another study reported as many as 68 substrates in cultured epithelial cell lines (Hemming et al., 2009). Hence, it is well possible that the muscle spindle defect caused by ablation of Bace1 involves other yet unknown substrates besides Nrg1, and that Nrg1 isoforms which do not depend on Bace1 activity participate in muscle spindle induction (as the authors rightly discuss). The complete rescue of the Nrg1 GOF phenotype by Bace1 ablation does not provide proof that Bace1-dependent cleavage of physiological levels of Nrg1 controls muscle spindle formation. I therefore suggest toning down the conclusions in all parts of the manuscript including the title ("Bace1 and Ig-containing Neuregulin-1 control formation and maintenance of muscle spindles").

2. Along similar lines, the authors should provide evidence that Ig-Nrg1β1 cleavage, or Ig-Nrg1, is impaired in Bace1-/- mice. In Figure 1I, the authors should add a blot showing what happens to Ig-Nrg1 expression in Bace1-/- mice. Alternatively, Bace1-/- cells should be transfected with Ig-Nrg1β1 to show that the cleavage is Bace1 dependent, or at least to which extent Bace1 is responsible for Ig-Nrg1β1 cleavage.

Other points
3. Figure 1D and 1E: Statistical analysis of homolateral and homolog coupling is missing.

4. Figure 2B: Percentage of co-localization should be quantified and Bace1 and Nrg1 co-expression should be shown as well in the muscle spindle.

5. Figure 2E-F: A blot showing Bace1 expression should be added. Moreover, a loading control, i.e. tubulin or actin, and a sample from non-transfected HEK293 cells should be shown.

6. Figure 3B: is the reduction in the numbers of muscle spindles significant among mutant genotypes, e.g. Bace1-/- vs co-IgNrg1 data?

7. Figure 3C: The increased survival of TrkC neurons in Ig-Nrg1β1ov mice might be dependent on a general trophic function of Nrg1 rather than on the increased number of muscle spindles. Indeed, in co-IgNrg1 mice although the numbers of muscle spindles are reduced, there is no effect on TrkC+ neuron survival. Authors should comment on this in the main text. Does Bace1 removal rescue TrkC+ neuron survival in Ig-Nrg1β1ov mice?

8. Calbindin staining intensity should be quantified for all mutants.

9. Figure 5C and 5D: Statistical analysis of homolateral and homolog coupling is missing.

10. Does long-term (30 days) in vivo treatment with the bace1 inhibitor impair locomotion?

1st Revision - authors' response 24 May 2013

Reviewer 1

Here, Cheret et al. report that BACE1 processing of IgNrg1β1 is required for formation, maturation, and maintenance of muscle spindles. The authors perform a series of experiments with BACE1$^{-/-}$ mice, BACE1 inhibitor treatment of wild-type mice, and conditional Nrg1 mice that are analyzed by gait analysis, biochemistry, and histology. The authors conclude that Bace1 plays a role in coordinated movement through regulating muscle spindle physiology and that an unwanted side effect of Bace1 inhibition for AD might be impaired coordination.

This is an interesting report with significant implications for both the AD and proprioception fields.

Comments:

1a) *In Figs. 1C-E and 5C-E, hindlimbs appear to be more severely affected than forelimbs in the various BACE1 and Nrg1 null mice. This might result from hypomyelination rather than spindle dysfunction, at least in the BACE1 knockout. Because hindlimb myelinated motor axons are longer than those of forelimbs, hindlimb motor axons would be expected to be more*

affected by hypomyelination than forelimbs.

Several mutations with moderate effects on motor coordination affect hindlimb coupling more strongly than forelimb coupling (whereas to our knowledge the reverse has never been observed); for instance the Swl mutants (Dync1h1 mutants, Chen et al, 2007), mice carrying a constitutively active EphA4^{EE} allele and double Nkx2.2$^{-/-}$Nkx2.9$^{-/-}$ mice (Egea et al, 2005; Holz et al) display such phenotypes. It might be possible that coordination deficits are less easily compensated on hindlimb levels. See also comment 1c) for the discussion of hypomyelination and gait analysis.

1b) *Although the authors measured g-ratios of sciatic nerve axons, representative images should also be included.*

We now provide representative images of sciatic nerves of P12 control and co-IgNrg1 mice in revised Fig. S5B.

1c) *In addition, the authors need to provide evidence that the gait disturbances are directly related to spindle dysfunction rather than hypomyelination in the mutants, particularly in the BACE1 knockout where hypomyelination is well established.*

Krox20^{cre}ErbB2$^{flox/flox}$ mice is another strain currently kept in our lab, which displays pronounced hypomyelination that is even stronger than the one observed in Bace1 mutants (control, Bace1$^{-/-}$ and Krox20^{cre}ErbB2$^{flox/flox}$: 0.68±*0.01*, 0.75±*0.01* and 0.80±*0.01*, respectively). We analyzed the behavior of Krox20^{cre}ErbB2$^{flox/flox}$ mice, and did not detect motor coordination deficits as assessed by the grip test or gait analyses. These data are now included in revised Fig. S2A-D and mentioned in the chapter *'Bace1 mutant mice display coordination defects'* on pg. 5 of the revised manuscript.

2) *Include age of adult mice and dose treated with BACE1 inhibitor in the text and the legend of Fig. 1I-K.*

We now mention age and doses of treatment in the Figure legend (revised Fig. 2A,B and its legend).

3) *Fig 3F-H: black bars are control mice but the key in G indicates white bars are controls.*

We thank the reviewer for pointing out this mistake, which was corrected in the revised Fig. 6C.

4) *For the conditional Nrg1 knockout coTxNrg1 (Fig 4.), evidence of lack of Nrg1 expression in muscle spindles is not shown but should be included.*

We now include qPCR data on the Nrg1 expression (or lack of it) in sensory neurons of coTxNrg1 mutant mice in revised Fig. 7B. IgNrg1 is not detectable in the muscle spindle or adjacent muscle tissue (cf. pictures of *in situ* hybridization for IgNrg1 transcripts in muscle, on pg. 4 of the present letter). Thus IgNrg1 provided by the sensory neuron induces and maintains the muscle spindle.

5) *Fig. 4D: How do the authors explain that the % of 1500-2000 µm spindles is significantly higher in coTxNrg1 mice? The % of spindles >2000 µm is clearly decreased, indicating spindle degeneration, but the increase in 1500-2000 µm spindles is confusing. It would be less confusing if the authors plot total number of spindles in each size range in addition to %.*

The reviewer is right to point out that the display of the data of the spindle length in coTxNrg1 mice we had used in the original submission was confusing. We displayed the distribution of spindle size as percentage of all spindles present. In the revised manuscript, we now display the distribution of the spindle size as absolute numbers in the Fig. 7G.

6) *The cre transgenic mice used in the study are not well described. Are the Deleter^{cre} animals mentioned in the materials and methods the same as the Wnt1^{cre} mice mentioned in the results?*

When is the cre transgene expressed in the Wnt1^{cre} mice? What promoter is used and what is the tissue expression profile for the cre-ER^{TM} mice?

We have rewritten the sections where we describe the Wnt1^{cre} and cre-ER^{TM} strains (pg. 9 & 12 of 'Results' section of the revised text, respectively) to improve the clarity. Similarly, more detailed description of the Deleter^{cre} and Wnt1^{cre} strains were included in the paragraph '*Animal strains and generation of IgNrg1^{flox} and IgNrg1$^{\Delta}$ alleles'* and of cre-ER^{TM} in the paragraph *'Ablation of Nrg1 expression during adulthood'*. Both can be found in Materials and Methods, pg. 18 & 19 of the revised manuscript.

7a) *Are the phenotypes the same for co-IgNrg1 and coTxNrg1 mice?*

The reviewer asks whether the phenotypes are identical in coTxNrg1 and in co-IgNrg1 mice. This is a question that is not easily answered for the following reasons:

The coTxNrg1 mice were bred and are kept in David Bennett's facility at Oxford, whereas co-IgNrg1 mice are kept in the MDC facility in Berlin. The first author (C.C.) observed both colonies and has voiced the impression that the behavioral phenotypes are amazingly similar. However, it was not possible to transfer the coTxNrg1 mice to Berlin for hygienic and ethical reasons during the revision time. The behavioral tests available in these two mouse facilities are not identical, thus we used gait analysis of mice on a moving belt in Berlin and a beam walk test in Oxford to assess impairments of motor coordination.

The behavioral similarities are: (i) Both strains (coTxNrg1 and in co-IgNrg1) display a pronounced homolateral coordination defect (hopping movements), and both tests used (gait analysis and beam walk test) demonstrate motor coordination deficits. (ii) Both mutants show deficits in the grid test, i.e. lose footing from the inverted grid much faster than their littermates controls (<25% of control hanging time).

In addition, similar changes in spindle number and morphologies are observed. Both strains show massively reduced numbers of muscle spindles, although there are subtle differences: the developmental mutants lose 90% of their spindles, while 60% of them degenerate in the inducible adult mutant. Similarly, the length of the remaining spindles is significantly reduced in both strains. We had routinely used calbindin staining in young mice to assess spindle diameters. However, calbindin expression is very low in the spindle of control 6-months old mice, and absent in the corresponding coTxNrg1 mutants. Because of this, we did not compare spindle diameters in late stages.

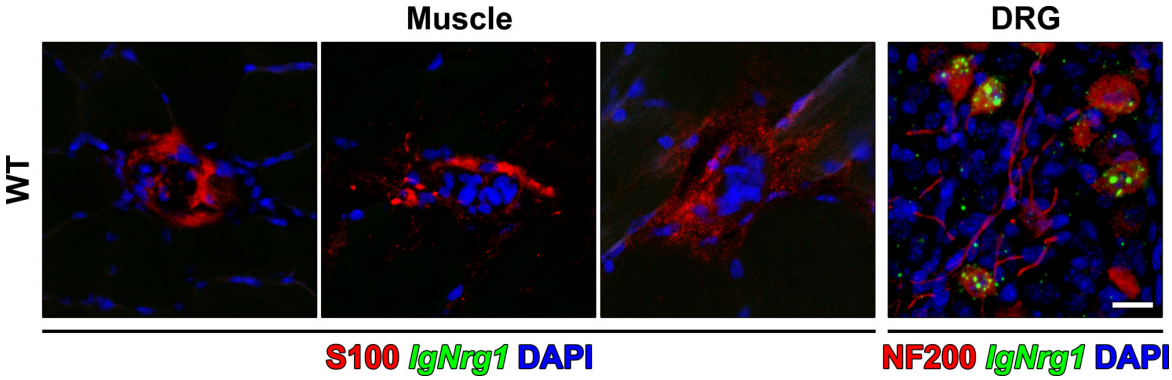
7b) A description of where the loxP sites are located in Nrg1 gene for the coTxNrg1 mice is lacking. It is assumed that coTxNrg1 is a total null of all Nrg1 isoforms, but this is not explicit. This is an important point, because there may be important differences between complete Nrg1 and IgNrg1-only null mice.

As already mentioned in the answer to comment 6 of this reviewer, we improved the description of the Wnt1^{cre}IgNrg1$^{flox/flox}$ (co-IgNrg1) and cre-ER^{TM}Nrg1$^{flox/flox}$ (coTxNrg1) strains in the 'Results' and 'Materials and Methods' sections, and clearly describe that tamoxifen-induced recombination in coTxNrg1 mice affects all Nrg1 transcripts (see the paragraph *'Maintenance of muscle spindles requires continuous Nrg1 signaling'* of the results, pg. 12).

8) *Abstract: "Our results should allow to monitor the effects of Bace1 inhibition in vivo." It is unclear exactly what the authors mean by this.*

We have removed the sentence in the revised manuscript.

Supplemental Data: Absence of IgNrg1 transcripts in muscle tissue



Detection of IgNrg1 transcripts (*in situ* hybridization, green) in S100+ (immunohistochemistry, red) muscle spindles of WT mice. The signal for IgNrg1 transcripts observed in NF200+ sensory neurons is shown for comparison. Neither extrafusal nor spindle-specific intrafusal muscle fibers express IgNrg1. Scale bar: 20 µm.

Reviewer 2

It was previously shown that Nrg1 is required in the early stages of muscle spindle development (Hippenmeyer et al., 2002). Between E14.5 and E18.5 proprioceptive afferents (Parvalbumin+ fibers) are required to induce the expression of early markers of muscle spindle differentiation. In $Isl1^{Cre}Nrg1^{flox/-}$ *mice myofibers fail to express these markers. It was further shown that Ig-Nrg1 isoforms are preferentially expressed by proprioceptive sensory neurons and are sufficient to induce muscle spindle differentiation in vivo, whereas CRD-Nrg1 isoforms not required for muscle spindle induction.*
The present manuscript by Cheret and coworkers confirms and extends these observations using a novel conditional Nrg1 mouse line in which the Ig-like domain of Nrg1 was targeted by Cre/loxP recombination. In addition, the present work reports on a new role of Bace1 in muscle spindle formation during development and in their maintenance in the adult. A tamoxifen-inducible Nrg1 KO was used to demonstrate a role for Nrg1 in maintenance of muscle spindles. Ablation of Bace1 and IgNrg1 produced similar gait abnormalities. In vivo overexpression of IgNrg1 in axons increased muscle spindle numbers, a defect which was rescued in the absence of Bace1, consistent with a model that Bace1-dependent cleavage of Nrg1 controls muscle spindle formation.
Overall, the technical quality of the data is excellent; the conceptual advance over previous work is decent, but not exceptional; the mechanistic evidence for Bace1-dependent cleavage of Nrg1 controling muscle spindle formation is modest.

Major points:

1) *Bace1 has many substrates in neurons. A recent study reported 34 substrates in primary embryonic neurons (Kuhn et al., 2012) and Nrg1 was not even included suggesting that this list is not complete. Another study reported as many as 68 substrates in cultured epithelial cell lines (Hemming et al., 2009). Hence, it is well possible that the muscle spindle defect caused by ablation of Bace1 involves other yet unknown substrates besides Nrg1, and that Nrg1 isoforms which do not depend on Bace1 activity participate in muscle spindle induction (as the authors rightly discuss).*
The complete rescue of the Nrg1 GOF phenotype by Bace1 ablation does not provide proof that Bace1-dependent cleavage of physiological levels of Nrg1 controls muscle spindle formation. I therefore suggest toning down the conclusions in all parts of the manuscript including the title ("Bace1 and Ig-containing Neuregulin-1 control formation and maintenance of muscle spindles").

We introduced several changes into the wording of the manuscript to accommodate the request of reviewer. For instance:

Title

Old: Bace1 cleavage of Neuregulin-1 controls formation and maintenance of muscle spindles

New: Bace1 and Neuregulin-1 (Nrg1) cooperate to control formation and maintenance of muscle spindles

Abstract

Old: Bace1-dependent shedding of IgNrg1 is required for formation and maturation of the muscle spindle

New: Our results assign to Bace1 a role in the control of coordinated movement through its regulation of muscle spindle physiology, and implicate IgNrg1-dependent processing as a molecular mechanism.

Last paragraph introduction

Old: […] these data demonstrate that Bace1-dependent processing of IgNrg1 […]

New: […] these data implicate Bace1-dependent processing of IgNrg1 in ontogenesis and long-term maintenance of muscle spindles.

2) *Along similar lines, the authors should provide evidence that Ig-Nrg1β1 cleavage, or Ig-Nrg1, is impaired in Bace1$^{-/-}$ mice. In Figure 1I, the authors should add a blot showing what happens to Ig-Nrg1 expression in Bace1-/- mice. Alternatively, Bace1$^{-/-}$ cells should be transfected with Ig-Nrg1β1 to show that the cleavage is Bace1-dependent, or at least to which extent Bace1 is responsible for Ig-Nrg1β1 cleavage.*

The reviewer asks us to show that endogenous Bace1 processes IgNrg1. We include in the revised manuscript a new panel (revised Fig. 3H,I) described in the chapter *'Bace1 processes Nrg1 isoforms'* (pg. 9). This blot shows the processing of transfected IgNrg1 in hippocampal neurons in the presence and absence of C3, a pharmacological inhibitor of Bace1.

The reviewer should note that only small proportions of sensory or hippocampal neurons express IgNrg1; the majority produces CRD-Nrg1. Unfortunately, all available antibodies recognize IgNrg1 as well as CRD-Nrg1. Because of the comparable overabundance of CRD-Nrg1, it is impossible to define full length and cleaved isoforms of endogenous IgNrg1 in extracts of differentiated neurons. Furthermore, we cannot generate enough primary sensory neurons for biochemical analysis, for instance after IgNrg1 transfection. We therefore used the more abundant hippocampal neurons, coupled with transfection of IgNrg1 and inhibition of endogenous Bace1.

3) *Figure 1D and 1E: Statistical analysis of homolateral and homolog coupling is missing.*

The statistical significance of the gait analyses presented in revised Fig. 1,2,4,S2 are now shown in Fig. S1A-C.

4) *Figure 2B: Percentage of co-localization should be quantified and Bace1 and Nrg1 (quantification) co-expression should be shown as well in the muscle spindle.*

These data are now shown in Fig. 3B of the revised manuscript. The vast majority of IgNrg1+ and of all DRG sensory neurons co-express Bace1 (99.8±*0.2*% and 93.7±*1.2*%, respectively). This quantification is now mentioned in the first paragraph of the chapter *'Bace1 processes Nrg1 isoforms'* on pg. 7 of the revised manuscript.

As described in the answer to point 4 of reviewer 1, IgNrg1 is not expressed in muscle spindles.

5) *Figure 2E-F: A blot showing Bace1 expression should be added. Moreover, a loading control, i.e. tubulin or actin, and a sample from non-transfected HEK293 cells should be shown.*

Loading control (calnexin), Bace1 expression and data from non-transfected HEK293 were added and are now included in the revised Fig. 3F.

6) *Figure 3B: is the reduction in the numbers of muscle spindles significant among mutant genotypes, e.g. Bace1$^{-/-}$ vs co-IgNrg1 data?*

The statistical significance of the quantification of muscle spindles number in the various mutant strains (revised Fig. 5A) is now shown in Supplemental Table II. We refer to the supplemental Table II in the chapter *'IgNrg1 isoforms are required for motor coordination'* on pg. 10 of the revised manuscript.

7a) *Figure 3C: The increased survival of TrkC neurons in IgNrg1β1^{Ov} mice might be dependent on a general trophic function of Nrg1 rather than on the increased number of muscle spindles. Indeed, in co-IgNrg1 mice although the numbers of muscle spindles are reduced, there is no effect on TrkC^{+} neuron survival.*

It was previously observed that proprioceptive neurons survive in the absence of muscle spindles. For instance, in Egr3 mutants, muscle spindles degenerate but the number of proprioceptive neurons is unchanged at birth (Tourtellotte et al, 2001). Similarly, muscle-specific ErbB2 mutant mice display severe degeneration of muscle spindles, but not of proprioceptive neurons (Leu et al, 2003).

A neurotrophin that is thought to play an important role in survival of proprioceptive neurons is NT3. NT3 is required for muscle innervation (and thus spindle induction since the spindle is not induced in the absence of proprioceptive innervation, see Kucera & Walro, 1990; Maier, 1997) and for survival of proprioceptive neurons (Farinas et al, 1994). Further, NT3 is clearly limiting, and heterozygous NT3 mutant mice contain about half the number of muscle spindles as control mice (Ernfors et al, 1994). In contrast, overexpression of NT3 in muscle rescues both muscle spindle formation and innervation in NT3$^{-/-}$ mice, and leads to the appearance of supernumerary spindles (Wright et al, 1997).

We think that due to the overexpression of IgNrg1, supernumerary muscle spindles are induced. These supernumerary spindles express NT3, resulting in broader NT3 expression and increased survival of proprioceptive neurons. This is analogous to effect of the transgenic overexpression of NT3 in muscle, which results in an increase of spindle numbers and survival of proprioceptive neurons (Wright et al, 1997).

A direct trophic function of Nrg1 on sensory neurons is not so likely; indeed, we have many years ago addressed such a potential role of Nrg1 in sensory neurons, and provided at that time evidence using chimeric mice that Nrg1 is not a trophic factor for sensory neurons *in vivo* (Riethmacher et al, 1997). Our data indicated that Nrg1 acts primarily on other cell types, e.g. Schwann cells or muscle, which in turn provide trophic support for neurons.

7b) *Authors should comment on this in the main text. Does Bace1 removal rescue TrkC+ neuron survival in IgNrg1β1^{Ov} mice?*

We discuss this increased survival (pg. 14, first paragraph of the chapter *'Functions of neuronally-produced Nrg1 isoforms during development and adulthood'* of the revised manuscript) and provide the numbers of proprioceptive neurons in Bace1$^{-/-}$ and Bace1$^{-/-}$ IgNrg1β1^{Ov} animals in revised Fig. 5C. In particular, the reviewer should note that in the absence of Bace1 (i.e. in the absence of supernumerary muscle spindles expressing NT3), IgNrg1 overexpression fails to increase the survival of TrkC+ proprioceptive neurons.

8) *Calbindin staining intensity should be quantified for all mutants.*

The quantification of calbindin immunoreactivity is shown in Fig. S6A of the revised manuscript.

9) *Figure 5C and 5D: Statistical analysis of homolateral and homolog coupling is missing.*

Statistical analyses are now included in revised Fig. S1A-C.

10) *Does long-term (30 days) in vivo treatment with the bace1 inhibitor impair locomotion?*

Long term *in vivo* treatment with the Bace1 inhibitor has similar effects on grip control and homolateral/homolog coupling as the Bace1 mutation. These data are now provided in the revised Fig. 2C-F and described in the second paragraph of the chapter: *'Bace1 activity is required to sustain muscle spindles and to maintain motor coordination'* on pg. 7 of the revised manuscript.

Reviewer 3

This manuscript showed that Bace1 processed IgNrg1β1 in in vitro proteolysis assay and suggests that Bace1-dependent processing of IgNrg1β1 is necessary for the formation and maturation of muscle spindles in mice. This work, using a variety of molecular and mouse genetic approaches, provides strong evidence for their claims. While no major concerns exist, several minor points need to be addressed.

1) *The authors stated that Bace1-dependent proteolysis is rate-limiting for spindle-inducing activity of IgNrg1. In experiments that showed increase of 50% of muscle spindles in IgNrg1β1^{Ov} transgenic mice would suggest that Bace1 is not rate-limiting. This point should be made clearer.*

The sentence was removed in the revised manuscript.

2a) The authors showed that ubiquitous deletion of Nrg1 in coTxNrg1 mice exhibit in addition to less number of spindles, outer capsule degeneration, a phenotype that is not observed in Bace1$^{-/-}$ mice, suggesting that Bace1 and Nrg1 act independently in controlling muscle spindle physiology.
Did coTxNrg1 mice show that same coordination defects as observed for Bace1$^{-/-}$ mice?

The comparison of the behavioral phenotypes of co-IgNrg1 and coTxNrg1 mice is also discussed above (answer to point 7 of reviewer 1).

2b) Could Bace1 and Nrg1 signaling pathway be acting in separate pathways?

The most severe phenotype is observed in muscle-specific ErbB3 mutants (K. Paulick and C.B., unpublished data), which contain no spindles (this and subsequent percentages refer to counting of spindles in P0 muscle). In co-IgNrg1 animals, we see a very severe reduction in spindles numbers (84% reduction), and remaining spindles are smaller and display fewer intrafusal fibers. Numbers (71% reduction) of muscle spindles are reduced in a pronounced manner in the compound Bace1$^{-/-}$IgNrg1$^{\Delta/+}$ mutants, but diameter and intrafusal fiber numbers are less strongly affected. Finally, the quantity of muscle spindles is reduced by 50% in both Bace1$^{-/-}$ mutants and Bace1 inhibitor-treated mice, and remaining spindles are smaller but contain correct numbers of intrafusal fibers in Bace1$^{-/-}$ mutants. This can be interpreted as graded changes in response to less and less Nrg1 signal received by muscle fibers in this series of mutants. The reduction in the signal is then translated into a more and more severe reduction in the numbers of spindles and an increasing severity of morphological changes of the spindle. We rewrote the discussion (Second paragraph of *'Functions of neuronally-produced Nrg1 isoforms during development and adulthood'*, pg. 15) to make this point clearer.

I think it is very unlikely that Nrg1 and Bace1 work in separate pathways. In our manuscript we assembled a multitude of data that indicate that Bace1-dependent cleavage of Nrg1 controls muscle spindle induction and maintenance. Maybe the best independent argument is the fact that we know of only two phenotypes in the peripheral nervous system that depend on neuronally-produced Nrg1, i.e. Schwann cell development/myelination and muscle spindle induction, and Bace1 mutants display in both cases an attenuated form of the phenotypes observed in Nrg1 mutants.

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